Isolation and Characterization of Soybean (*Glycine max*) Lectin

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Submitted by

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CERTIFICATE

This is to certify that the thesis entitled "**Isolation and Characterization of Soybean(*Glycine max*) Lectin**" which is being submitted by **Mr. Chandra Sekhar Bhol**, Roll No. **410LS2044**, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by him under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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DEDICATED TO

MY BELOVED PARENTS

AND FAMILY

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At the end, I bow down my head to my Gurudev and the almighty whose omnipresence has always guided me and made me a greater person.

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This is to certify that the Project Work entitled "**Isolation and Characterization of Soybean (*Glycine max*) Lectin**", submitted to National Institute of Technology is a faithful record of bonafide and original research work carried out by Mr Chandra Sekhar Bhol, has been approved by the student advisory committee.

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I do hereby declare that the Project Work entitled "**Isolation and characterization of Soybean(*Glycine max)* Lectin**", submitted to the Department of Life Science, National Institute of Technology, Rourkela is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. Sujit Kumar Bhutia, Asst. Professor, Department of Life Science, National Institute of Technology, Rourkela, Odisha.

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LIST OF ABBREVIATIONS

°C	**:**	**Degree Celsius**
Min	**:**	**Minute**
H	**:**	**Hour**
M	**:**	**Molar**
mM	**:**	**Mili molar**
L	**:**	**Liter**
mL	**:**	**Milli Liter**
Kg	**:**	**Killogram**
gm	**:**	**Gram**
mg	**:**	**Milligram**
µg	**:**	**Micro gram**
µm	**:**	**Micro meter**
%	**:**	**Percentage**
OD	**:**	**Optical Density**
EDTA	**:**	**Ethylene diamine tetra acetate**

ABSTRACT:

Plant lectins have a wide spectrum of biological significance. In this thesis work the isolation and characterization of the lectins(SBL) from the Soybean(*Glycine max)* have been reported. The lactose binding lectins were purified from the Soybean seeds by performing affinity chromatography using a lactamyl sepharose 4Bcolumn. Affinity products were dialysed against PBS and SDS-PAGE was done to identify the fractions' molecular weight. Furthermore, haemagglutination assay was performed for crude, 20% cut, 60% cut, 90% cut, 60% affinity and 90% affinity portion using AB blood group type. This work explored different techniques of protein (lectin) purification and describing their characteristics by doing the haemagglutination assay.

Keywords: Soybean lectin, SBL, Affinity chromatography, SDS-PAGE, haemagglutination assay.

1. INTRODUCTION:

The word "Lectin" has been derived from the Latin word which means "I choose". Because lectins are very specific to the site to which it binds. Lectins are carbohydrate-binding proteins which bind to glycoproteins, glycolipids, and also polysaccharides (Goldstein and Hayes, 1978) which mediates various kind of biological processes by binding to different sugar moiety (Lis & Sharon, 1998; Vijayan & Chandra, 1999). Lectins were characterized for their agglutination properties with erythrocytes of human and other animals, is the easiest and most convenient method of detection of lectin activity (Laija et al.,2010). Lectins are also called agglutinins because when it binds to cell surfaces results in agglutination reactions. Lectins are highly diverse in structure, molecular weight, composition, and number of sugar binding sites present per molecule (Laija, et al.2010). Lectins are a well known proteins which has highly specific carbohydrate-binding. Lectins have non-immune origin and can binds to oligosaccharides, glycoproteins, glycolipids and different polysaccharides without changing their covalent structure (Kocourek & Horejsi, 1981; Barondes, 1988). The lectins were first described by Stillmark in 1888 when he was working with castor bean extracts and showed agglutination reaction. On the basis of their carbohydrate binding specificity, lectins can be classified into many groups such as mannose-binding, glucose-binding, galactose-binding, N-acetylglucosamine- binding, N-acetylgalactosamine- binding, fucose-binding, and sialicacid-binding.

Lectins are widely distributed in nature and found in all forms of life including plant products such as fruits, vegetables but nuts, grains, beans and seeds contains high lectin amount (Lis and Sharon, 1986).Researchers has great interest and lectins has been studied and isolated from various sources including plants , animals, fungi, lichens and bacteria(Liener1976; Hapner and Robins 1979; Damjanov 1987; Sharon and Lis 1989). It has been reported that lectins have been found mostly in seeds of Legumes and other plant species (Liener 1976; Pueppke 1979; Quinn et al.1987). The interest on plant lectin study is due to their high specificity on carbohydrates (Willy J. Peumans et al.,2010). Lectins have also been isolated from vegetative tissues of plants like leaves, stems, barks, and roots(Callow 1975; Goldstein and Haye 1978; Quinn and Etzler1987). Lectins are also suited for analysis and isolation of animals and human glycocojugates. The mature seed contains about 3% of the weight of it (Laija et al.,2010). The plant lectins are stable proteins which can be characterized without affecting their sugar binding properties.

The banana lectin has been isolated from *Musa paradisiaca* and characterized in 1990 (Koshte et al.,1990). The dimeric structure of lectin was shown to be mannose-specific

(Singh,D.D., 2004). The snail *Helix pomatia*, contains large amounts of a lectins which agglutinates with human type A erythrocytes (Uhlenbruck & Prokop, 1966). It has been reported (Vasta, 1992) that this lectin, which aggregates in the snail albumin gland (Prokop et al., 1968), plays a major role in the protection of eggs as well as developing embryos against bacterial and fungal infections. The lectins from legume and cereal has altered the microflora present in the gut (Liener, 1986; Pusztai et al. 1993b;), causes inflammation (Liener, 1986; Pusztai et al. 1993b) and increases the intestinal permeability(Greer et al. 1985) which also helps in the translocation of gut pathogens to the periphery. When kidney bean lectin (PHA) given in high doses to conventional rats proved lethally toxic(Wilson et al. 1980), but is nontoxic for the germ-free animals (Rattray et al. 1974). Lectin activity has been demonstrated in wheat, barley, oats, maize (Liener, 1986). Maize, like wheat, alters intestinal epithelial structure as well as function. (Mehta et al. 1972). The biological activities of cereal lectins are highly similar because they are closely related to one another both structurally and immunologically (Peumans & Cammue, 1986). The root extract of *Arachis hypogea*(groundnut) and its seed lectin were found to stimulate the synthesis of exopolysaccharide and capsular polysaccharide of the micro symbiont cowpea Rhizobium strain. When lectins of Abrus was treated with rabbit erythrocytes and injected in the peritoneal cavity of mice showed agglutination reaction.

The biological activities like anti-tumor, anti-proliferative, immune potentiating, antibacterial, antifungal, anti-insect, and antiviral activities have been found in lectins. Microbes carry lectins, which help them for attachment to the host cells. The human body contains lectins at various places such as, on the vascular endothelial linings(selectins) in order for blood cells to escape into the tissues; in to capture microorganisms, and as substances called opsonins, that coat foreign antigens, making them more susceptible to phagocytosiss. The screening of number of breast cancers against lectins shows different specificities (Leathem & Brooks, 1987). Lectin associated haemagglutination assay and the ability of different oligosaccharides to act as inhibitors was determined by the microtitre 'V' plates by using rabbit erythrocytes (Lechaire & Barondes, 1978). The lectins consumption disturbs normal growth in humans as well as in other animals. It has been reported that the lectins influences the nutrient intake (Liener, I. E.,1986). Lectins causes morphological injury in the small intestinal mucosa due to its adhesion to the mucosal surface. But the injury can be prevented by the simultaneous administration of saccharides having specific affinity for the lectins or by treatment of the foods containing lectins

with heat. Lectins are found commonly in most legumes and their toxic effects have been seen. (Liener, I. E. 1986). Pusztai et al. reported that beans having higher content of lectins causes most serious damages to the luminal surface of intestine in rats compared to those having lesser lectin contents. An inhibitory effect of lectins on the activity of peptidase as well as disaccharidase of enterocytes has also been reported (Oliveira, A. C.,1989, Kim, Y. S., Brophy, E. J. & Nicholson, J. A.,1976). Besides playing a role in innate immunity mannose binding lectin (MBL) helps in cellular defenses such as phagocytosis, and pattern-recognition receptors that activates pro-inflammatory signaling cascades. Lectins isolated from bean species has influenced the intestinal structure and function negatively (Liener,I.E.,1986) leading to diseased situations. Some lectins show anticancer property in vivo as well as in vitro, as a result they can be used as therapeutic agents for tumor inhibition as it causes apoptosis. Lectins has affinity to bind with ribosomes as a result it inhibits protein synthesis. Lectins can decreases the telomerase activity and suppresses angiogenesis. Distinction between a malignant cell and a normal cell has been done by using lectins (Sharon and Lis 1989, 2004). Lectins has the property which modify the cell cycle by inducing non-apoptotic G1-phase accumulation mechanisms, which arrests G2/M phase cell cycle and do apoptosis and also has the ability to activate the caspase cascade. Plant lectins has the capacity in cell separation and bone marrow transplantation (Reisneret al., 1978, 1983). Introduction of plant lectins in the form of microarrays as a unique means for high throughput analysis of protein glycosylation (Rosenfeld et al., 2007) and profiling the global changes in the mammalian and bacterial (Hsu et al., 2006) cell surface glycomes.

Lectins can be used as probes for the characterization and isolation of simple and complex sugars (Rudiger and Gabius, 2001). Lectins can be used in immunological studies as a tool (Moreira et al., 1991). Dietary lectins can cross the gastrointestinal barrier and enters the circulation intact (Pusztaiet al.1989), and also be able to interact with synovial tissues directly. Lectins has the tendency of alternating interleukin production which affects the body immune system. Various plant lectins has the tendency to bind with the intestinal mucosa which disturbs the functions of intestine and may causes enlargement of pancreas (Pusztai and Bardocz, 1996). Plant lectins plays a vital role to defense itself from the predators is not new. Lectins are used in the activation of lymphocytes and for induction of proteins like enzymes, interleukins or cytokines (Kilpatrick, 1991). To determine the 3-dimensional structure of carbohydrate binding site of lectins NMR and XRD are used. Lectins have many applications starting fom

identification of microorganisms to cancer research. Mitosis (cell division) can be enhanced with pokeweed lectin (PWA). Lectins has been used as carriers in drug delivery such as delivery of chemotherapeutic agents. The lectins isolated from different plant species often varies greatly in their molecular structure and specificity. Many legume species contain proteins which are clearly related to the lectins lacking carbohydrate binding activity. A well-known example of this group of proteins are the *Pkaseolus vulgaris* arcelins and the a-amylase inhibitor (Mirkov et al., 1994). Biochemical, cellular, evolutionary, molecular, and physiological arguments indicate that lectins have a role in plant defense. The toxic nature of plant lectins and its effects on animals and humans have been obtained from feeding experiments with purified PHA and also observed from accidental poisoning of humans by taking raw or insufficiently cooked beans.

2. REVIEW OF LITERATURE:

The soybean or soya bean (*Glycine max*) is a legume species native to East Asia, which is highly cultivated for its edible seed. The plant has been classified under oil producing plant for its edible seed rather than for its pulse by the Food and Agricultural Organization (FAO). Soybean is an annual legume categorized under Fabaceae family. Soybean is one of the most important bean among all in the world which provides vegetable protein for millions of human and ingredients for thousands of chemical products. It is most nutritious and easily digested food of the bean family. The soybean is considered as one of the richest and cheapest sources of protein. It is a staple in the diet of humans and animals in different corners of world today. The seed contains 17 percent oil and 63 percent meal, 50 percent of which is protein. Soybean is aa good source of protein for diabetics as it contains no starch. Globally, the most important feed grain legume is soybean (*Glycine max*), with a total production of 216,144,262 tonnes and harvested area of 94,899,216 hectors (Faostat, 2009).

Today, the world's top most producers of soybean are USA, Brazil, Argentina, India and China. Approximately 85 percent of the world's soybeans are processed, or "crushed," annually for the production of soybean meal and oil and 98 percent of the soybean meal that is crushed is further processed for preparation of animal feed. About 95 percent is consumed as edible oil; the rest is used for industrial products such as in the production of fatty acids, soaps and biodiesel.

Soybean is an agricultural crop of great importance as many important proteins and non-proteinaceous compounds have been isolated. Soybean (*Glycine max*) seeds are known to contain different proteins which have anti nutritional and/or toxic effects, such as soybean agglutinin (an *N*-acetylgalactosamine-specific lectin).

Soybean lectin isolated from *Glycine max* is a carbohydrate binding protein highly specific to terminal non reducing N-acetyl-D-galactosamine but less to D-galactose. Presence of galactose during biofilm formation had various effects in the presence or absence of SBL.(Julieta Pérez-Giménez, et al. 2009). Soybean lectin has a vital role in the initial recognition of *Rhizobium japonicum*by the plant, which leads to a strain-specific, nitrogen-fixing symbiosis (Bohlool& Schmidt, 1974). Supplementation of soybean lectin (SBL) in diets resulted in a decrease in the activity of trypsin while protein levels and amylase activity increased in the pancreatic juice (Hemalatha, C., 2011). Inhibitory effects of soybean proteinisolate (SPI) and

soybean lectin on the intestinal absorption of nonheme iron were investigated by in vivo studies in rats. The soybean lectin has inhibitory effect on iron absorption (Sanae Hisayasa et al.). Soybean seeds from different cultivars have dissimilar protein products. The trypsin inhibitors of Bowman-Birk type is produced by yellow soybean (Losso JN., 2008). But the Chinese dull black soybean produces Kunitz type trypsin inhibitor (Lin. P,et al. 2008). The lectin of little black soybean was stable only up to 40 ºC for 20 min, rather the small glossy black soybean lectin was stable up to 70 ºC for 30 min. SBL isolated by the gel filtration chromatography gave a single peak but when subjected to electrophoresis in native conditions, moved as a single band and showed a native molecular mass of 110 kDa and under denaturing conditions the lectin gave a single band at a position of 30 kDa.

Soybean lectin eliminates cancer cells from the marrow and to decrease the risk of graft-versus-host disease soybean lectin is used for removal of the marrow of T cells during treatment of acute lymphoblastic leukemia (Reisner Y, 1983). The blood glucose levels was decreased in both diabetic induced and non-diabetic rats when fed with soybean lectin (Hemalatha, C., 2011). Pancreatic protein, DNA and RNA contents were increased by soybean lectin in both diabetic and non-diabetic rats (Pusztai,A. (1991)., Pusztai,A. (1993),Grant G. et al 1997.). The soybean lectin is a inhibitor of HIV-1 reverse transcriptase and proliferation of breast cancer MCF7 cells (Peng Lin, et al, 2008). It has been reported that soybean diet decreases chances of breast cancer and prostate cancer. Protease inhibitors present in soybeans reduces protein digestion, induce pancreatomegaly and enhance chemically induced pancreatic tumors in some animals (Grant et al, 1990). The trypsin or chymotrypsin inhibitor (Bowman-Birk inhibitor) present in soybeans has been studied as an anticancer agent (Kennedy, 1995). The lectins isolated from Chinese black soybean has the capacity to inhibit HIV-1 reverse transcriptase and inhibit HIV replication. The anti proliferative and anti-tumor activities of lectins were well studied (Wong JH, Ng TB., 2005). Black soybean lectins manifest weaker mitogenic activity than Con A toward splenocytes. An anti-tumor action mechanism of soybean lectins has property of reduction of tumor cell proliferation, apoptosis. And the induction of tumor-specific cytotoxicity of macrophages. That's why tumor cells are more susceptible to attack by macrophages after treatment with lectins. In addition lectins exert an immune modulatory effect on altering interleukins production (de Mejia EG et al 2003.).

Soybean is a unique dietary source of isoflavones which displays a diversity of biological activities and reduces the risk of some unrelieved diseases. Numerous clinical studies have disclosed the significance of consumption of soybean protein due to presence isoflavones to achieve a hypo cholesterolemic effect (Lichtenstein, 1998).Due to high content of isoflavones which supposedly diminish the risk of diseases like cancer, osteoporosis, cardiovascular disease and also alleviate menopausal symptoms. Addition of isolated soybean protein with isoflavones in the diet of postmenopausal women causes a decline in the incidence of hot flashes (Albertazzi et al., 1998). Improved cognitive ability was observed in postmenopausal women after intake of soybean extract containing isoflavones.

Among the anti-nutritional factors present in soybean seed, the main ones are protease inhibitors – Kunitz trypsin inhibitor (KTI) and Bowman-Birk inhibitor, and lectins. Protease inhibitors represent 6% of the protein present in soybean seed.80% of the trypsin inhibition is caused by KTI, which strongly inhibits trypsin and therefore reduces food intake by diminishing their digestion and absorption. Another effect of KTI is the induction of pancreatic enzyme, hyper secretion and the fast stimulation of pancreas growth, hypertrophy and hyperplasia. That's why raw soybean cannot be used for feeding monogastric animals.

Soybean agglutinin (SBA) causes the atrophy of the microvilli, reduces the viability of the epithelial cells and increases the weight of small intestine because of hyperplasia of crypt cells (Grantet al., 1987; Pusztai et al., 1990). Soybean lectin is resistant to inactivation by dry heat treatment (A. Mikić et al.,2006). The lectin gene represents one of the repertoires of seed protein genes which is highly regulated during the soybean life cycle. This gene does not contain introns, directs the synthesis of a 1.1-kilobase (kb) mRNA that accumulates and decays during embryogenesis, and is regulated in part at the transcriptional level. Lectin gene is highly expressed during soybean embryogenesis and that at its highest level lectin mRNA constitutes 0.75% of the embryo mRNA mass (Okamuro, J.K.,et al., 1986). Thermal treatment is the oldest and most frequently used method for modification soy proteins but the purposes of the thermal modification are different. Thermal treatments of soybean reduces protease inhibitor activity, by eliminating lipoxygenase and volatile compounds that induce unwanted flavor, improve specific functional characters which has no adverse effect in nutrition. Also, heating increases digestibility of soy proteins. Heating soy proteins above 70°C causes dissociation of their quaternary structures by denaturing its subunits, and promotes the formation of protein

aggregation via electrostatic, hydrophobic and also by disulfide interchange mechanisms. The 7S and 11S globulins have different thermal susceptibility. Trypsin inhibitors found in raw soybeans cause growth inhibition, pancreatic hypertrophy and hyperplasia in experimental animals (Liener, I. E., 1996). Kunitz inhibitor consists of181 amino acid residues with two disulfide bridges; one of these bridges is essential for inhibitor activity. The soybean genotypes lacking SBA were found Pull et al (1978). Soybean contain high amount of oligosaccharides, consisting mainly of raffinose and stachyose which are poorly digested and have been implicated as causes for the poor utilization of energy from soybean meal fed to poultry (Lesake et al., 1995). Soybean contains anti-nutritional factors which are physiologically active compounds with small or unknown effects including antivitamins, isoflavones, saponins and tannins (A. Mikić1,et al, 2006). The mitogenic activity of soybean lectin toward human and murine lymphocytes is enhanced after polymerization by physical or chemical means (Lotan R. et al.1973.). Natural suppressor cells from the spleen and cyclo-phosphamide generated suppressor cells react specifically with soybean lectin and can thus be isolated by agglutination from the bone marrow (Hoskin D.W, et al 1992 ; Brooks et al 1993). Soybean lectin reacts with and eliminates cancer cells from the marrow (Reisner Y.,1983). Black soybean lectins has mitogenic activity towards splenocytes and their antiproliferative activity toward tumor cells (Reisner Y.1981;Lotan R. et al 1973). Soybean lectin reportedly reacts preferentially with some rumen fungi (Baintner K, 1993).

The lectins purified from soybean showed equal agglutination with all blood groups of human beings i.e. A, B, AB and O (Bashir H. et al. 2010). Soybean (Glycine max) has haemaglutinating activity and contains a protein which reacted with antibodies directed against soybean seed lectin was the same as root lectin (Gatehouse and Boulter 1980). The lectin activity was determined by measuring hemagglutination assay (HAG) the method of Nowak et al. 1976. The hemagglutinating activity varies in different soybeans. Soybean lectin mediated agglutination of sheep red blood cell (SRBC) shows (Reisner et al., 1983a) differential effects on human marrow cell suspensions (Reisner et al., 1983).

3. OBJECTIVES:

- Isolation of Lectin from Soybean (*Glycine max*)
- Measurement of concentration of proteins
- Haemagglutination Assay
- SDS-PAGE

4. BIOLOGY OF THE SPECIES:

Fig.1:Soybean Plant

Fig.2: Soybean Seed

4.1 CLASSIFICATION:

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Rosidae

Order: Fabales

Family: Fabaceae

Genus: Glycine

Species: max

4.2 COMMON NAMES:

English Name: Soy beans

Japanese Name: Edamame

Chinese Name: M*aodou*

Pakistan Name: Photas

Indian name: Soya

4.3 PLANT DESCRIPTION:

4.3.1 MATURE PLANT: Soya bean grown for seed production is an annual, leguminous plant, normally bushy and erect i.e. upright growth habit. Usually plant height varies from 40 to 100 cm, and plants are much branched with well developed roots. Each plant produces a number of small pods containing one to four round, usually yellow to black seeds. It has a round hairy stem with branches. Leaves are alternate, vary in shape, are hairy in some varieties, are normally trifoliate, with the three ovate or lanceolate leaflets borne on a long petiole, and they stipulate.

4.3.2 CLIMATIC CONDITION: Yields are adversely affected as temperatures rise above 30 °C, while temperatures below 13 °C for long periods during flowering stage inhibit flower and seed formation. Although 25 °C can be considered the overall optimum temperature for all growth stages, the response of the soybean differs at various growth stages. At planting time, soil temperatures must preferably be in the region of 15 °C in order to stimulate germination. Rainfall of 500 to 900 mm is required for better yields and better seed quality, depending on growth conditions. Deep, well-drained soil with a fine but firm seedbed that is high in fertility and has good water-holding capacity is needed for good soya bean yields.

4.3.3 DISTRIBUTION: Soya beans are indigenous to Manchuria, China. The soybean plant is an annual plant native to Southeast Asia including Indonesia, the Philippines, Japan, Vietnam, Thailand, Burma, Malaysia, Nepal and north India.

5.MATERIALS AND METHODS:

5.1 CHEMICALS **:**

Sodium hydroxide (NaOH), Sodium carbonate (Na_2CO_3), glycine,Cupper sulphate($CuSO_4$) , Potassium sodium tartarate ($KNaC_4H_4O_6$) were purchased from SRL, Sisco Research laboratories Pvt. Ltd., Mumbai. Acrylamide, bisacrylamide, Ammonium persulphate (APS), Sodium dodecyl sulphate (SDS), N,N,N',N'-tetramethyl enediamine(TEMED), Bovine serum albumin(BSA), Tris were purchased from Sigma Aldrich, USA. Folin-Ciocalteau phenol reagent, Potassium Dihydrogen Phosphate (KH_2PO_4), Potassium hydrogen phosphate (K_2HPO_4) were purchased from S.D. fine chem. Ltd., Mumbai. Acetic acid, Bromophenol blue, agarose were purchased from Himedia, Mumbai. Glycerol was purchased from RANKEM Pvt Ltd. Ethanol purchased from Trimurty Chemicals, India. Pre stained molecular weight marker was purchased from Bio-Rad, India. Methanol, Silver nitrate, Sodium thiosulphate were purchased from Nice chemicals Pvt.Ltd. India.

5.2 SAMPLE COLLECTION:

The Soybean(*Glycine max)* seeds were collected for isolation and purification of Lectins from a local food store situated at Rourkela market, Odisha. The chemical used in the study are supplied by Hi-media and Sigma Aldrich.

5.3 SEED COAT REMOVAL:

Soybean seeds were taken and grinded in a mixer for removal of seed coats and 50gms of uncoated seed were taken for the study. The uncoated seeds were soaked in PBS for overnight. Then the seeds are grinded with minimum volume of PBS and the pastes were collected in 50 ml centrifuge tubes and were centrifuged by the eppendorf centrifuge 5430R with 7500rpm, at 4^0 c for 20 mins. The supernatant were taken after centrifuge and measured by a measuring cylinder. Some supernatant were stored in an eppendorf tube as crude at 4^0 c and the remaining were taken as salting out Process.

5.4 SALTING OUT:

When the ionic strength of a protein solution is increased by adding salt, the solubility decreases, and protein precipitates. The salt molecules compete with the protein molecules in binding with water. The concentration of salt requires for precipitation of the protein out of the solution is varies greatly in different proteins. It is also used to concentrate dilute solutions of proteins. Ammonium sulphate salt was taken in the salting out process. Crude were taken for 20% cut off in the salting out process. According to the salt chart ammonium sulphate were added to the crude by pinch wise and continues stirining was done by magnetic strirer.

Fig.3:Salting out with Magnetic Strir

Then the sample was stored for overnight at 4^0 C and in the next day the sample was taken for centrifuge, then supernatant and pellet was collected. The amount of supernatant was measured by a measuring cylinder and taken for 60% cut off. The supernatant was taken and ammonium sulphate salt was added in pinch wise and continues stirining was done by magnetic strirer.

Similarly like 20% cutoff and 60% cutoff supernatant was collected and measured ammonium salt was added for 90% cutoff and stored at 4C overnight. The pellet was collected after centrifuge and undergo dialysis in PBS for 3-4 days.

5.5 PREPARATION OF LACTAMYL SEPHAROSE 4B AFFINITY MATRIX:

5.5.1 EPOXY ACTIVATION OF SEPHAROSE 4B:

4gm of lactamyl sepharose 4B matrix was washed with 6ml distill water and mixed with 2.6ml of 2N NaOH and 0.66ml epichlorohydrin were added so that the final concentration of the various components were 30% v/v sepharose, 5% epichlorohydrin, 0.4 M NaOH. It was cover with aluminum foil and incubated at 40^0c for 2h with shaking. It was then transferred to a glass filter funnel and the gel was washed with 500 ml of distilled water.

5.5.2 PREPARATION OF AMINO SEPHAROSE 4B:

Epoxy activated sepharose 4B was suspended in 1.5 volume of concentrated ammonia solution i.e. 6 ml. The suspension was incubated at 40 c for one and half hour. It was then again transferred to a glass filter funnel and the gel was washed with distilled water.

5.5.3 COUPLING OF LACTOSE WITH AMINO SEPHAROSE 4B:

4gms of Suction dried amino sepharose 4B was suspended in 3ml of 0.2M K_2HPO_4 buffer, which was contain 51mg $NaCNBH_3$ and 104 mg of Lactose. The Suspension was incubated at room temperature for 10 days with occasionally shaking. The free amino groups which remained in the gel were acetyled by adding 2 ml of acetic anhydride. The suspension was incubated in the room temperature for 1 hour. The lactamyl sepharose 4B thus obtained was subcequently washed with distilled water, 0.1 M NaOH, distilled water and 10 mM PBS subsequently. It was stored in distilled water with traces of sodium azide at 4C.

5.6 AFFINITY CHROMATOGRAPHY:

The lactamyl sepharose column washed by PBS solution (pH7.2) and O.D of the washed PBS was measured at 280nm. When the OD value decreases and tend to zero then the Protein sample of 60% cut off was passed through lactamyl sepharose beads and the elute sample was collected and its O.D was determined at 280nm.

Lactatamyl sepharose beads were again washed with PBS solution (pH7.2) and the O.D of the washed PBS was measured at 280nm. When the OD value decreases and tends to zero then 20ml 4M laltose solution was loading on lactose sepharose beads and O.D of the eluent was measured at 280nm. The eluent was collected for dialysis in PBS (pH 7.2) and stored at 4^0c for 1day. Same Procedure was followed for the 90% cutoff. In 90% cut off 30 ml of Lactose solution was passed through the Lactose sepharose 4B column.

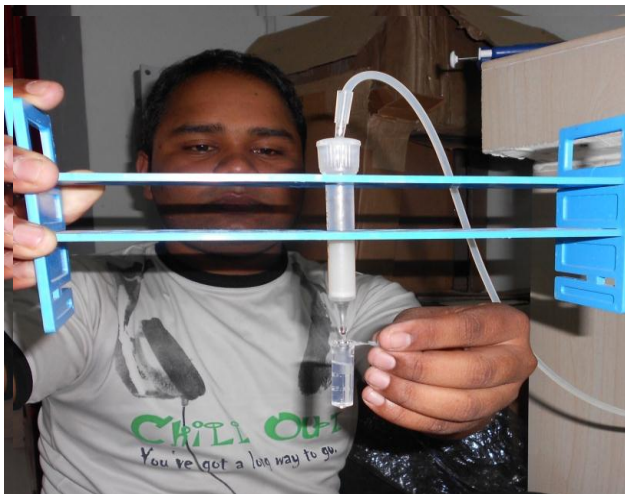

Fig.4:Lactamyl sepharose 4B Column

5.7DIALYSIS:

Dialysis was done against PBS at 4^0C of 20% cut off, 60% cut off, 90% cut off and 60% Affinity and 90% Affinity samples.

Fig.5: Dialysis of sample with PBS

5.8 DETERMINATION OF CONCENTRATION OF PROTEIN:

The concentration of crude, 20% cut, 60% cut, 90% cut, 60% affinity and 90% affinity were measured by Lowry method using bovine serum albumin as the standard protein (Lowry et al., 1951).

5.8.1 LOWRY'S METHOD:

REAGENT A=Sodium hydroxide(0.5%)

Sodium carbonate(2%) make it upto 1 litre

REAGENT B1=1% Copper sulphate

REAGENT B2=2% Sodium potassium tartarate

REAGENT C=A:B1:B2=100:1:1

BSA STANDARD=1mg/ml

Folin ciocalteau's reagent=1N (5 ml solution +5 ml distill water)

Take different concentration of BSA solution from stock solution and add distill water to it and made up to 2ml.*Abrus* protein taken unknown quantity dissolved in 1ml distill water.And add reagent C of 5 ml and protein of 0.5ml. Mixed properly and incubate for 10 mins. Then 0.5 ml of Folin reagent was added and incubate for 30min. Take OD at 750nm.

5.9 PREPARATION OF HUMAN ERYTHROCYTE:

Healthy human venous blood (4-5ml)was collected in a 15 ml tube to which the anticoagulant EDTA was previously added.

5.10 HAEMAGGLUTINATION ASSAY:

1ml blood sample was centrifuged in 2ml microtube at 1000 rpm for 5min at room temperature by Eppendrof mini spin. The the pellet was collected and was added 10ml of PBS. The mixture of blood and PBS was centrifuged at 1000 rpm for 5min at room temperature. After centrifuge the pellets were collected and 100µl of pellet was added to10ml of PBS solution (pH 7.2). The haemagglutination activity of Soybean lectin was detected when blood erythrocytes were added to it. The assay was carried out in a 96 well round bottom microtitre plate. The first well of each row was served as positive control to which 100µl of normalized sample and 100µl

of blood was added and the last well served as negative control since it contain 100µl of blood and 100µl of PBS solution. Between the positive and negative control each well contains blood, PBS and lectins. First of all 100µl PBS was added to all the wells. Then 10µl of normalized crude was poured to the first well and it was serially diluted till the negative control. Similar procedure was followed for the other samples. Finally 100µl of processed blood sample was poured to each well. After that the plate was placed in a plane surface without disturbing it. After two hours the haemagglutination assay result was observed.

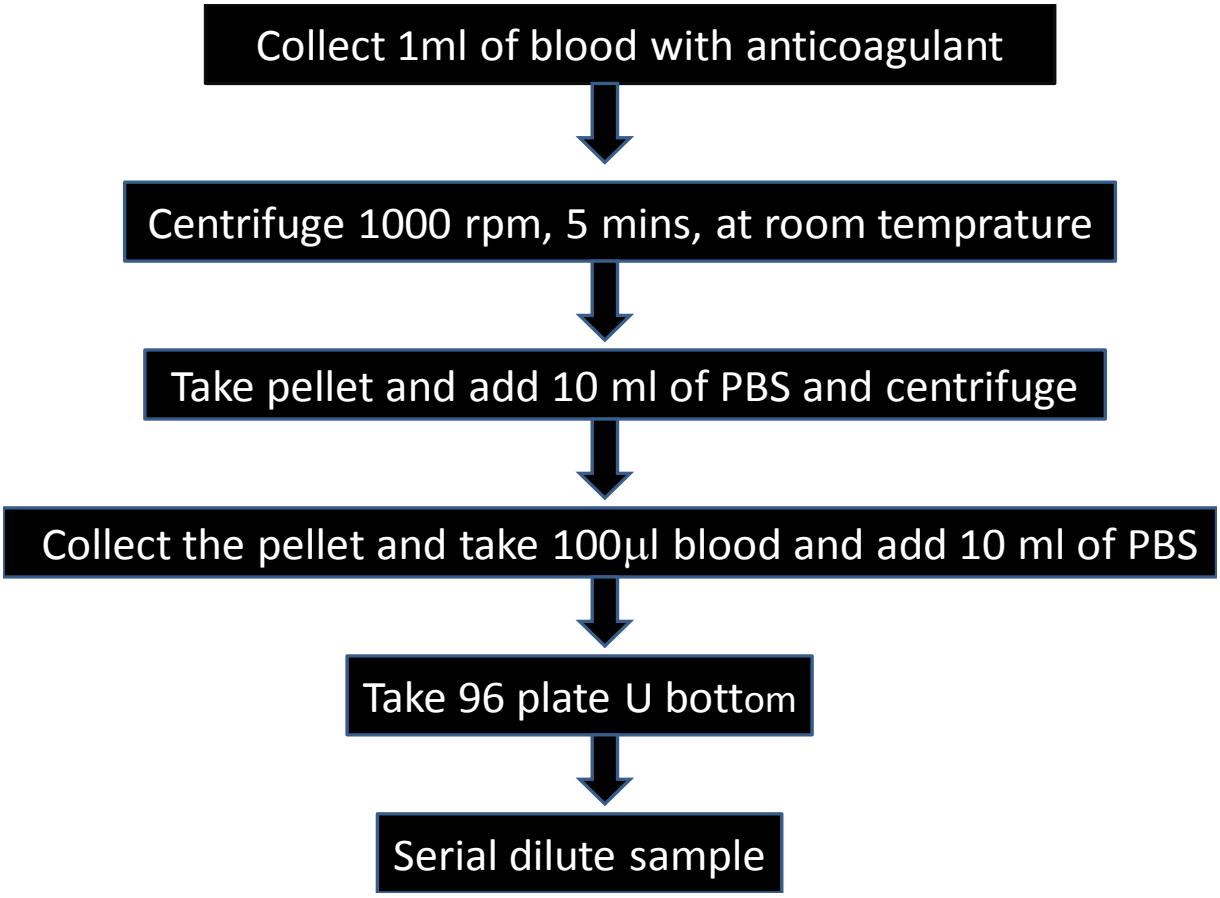

Fig.6 : Protocol for Haemaglutination assay

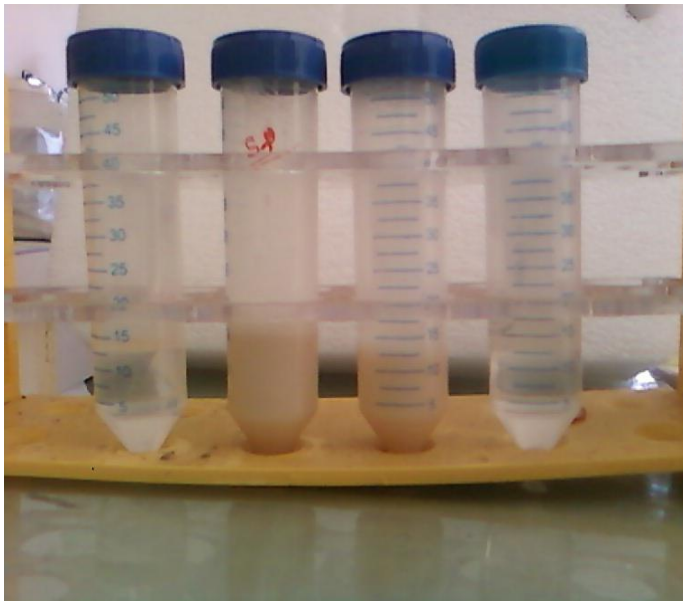

Fig.7: Protein Samples

5.11 SDS-PAGE:

The molecular mass of the subunits of the lectins was estimated by SDS-PAGE(12%). The poly acrylamide gel electrophoresis was done according to the protocol given in the Book "Molecularcloning" by Sambrook& Russell. The mixture of 10µl of sample, 10µl of sample loading buffer were added to the well. Crude, 60%, 90%, 60% affinity and 90% affinity sample was allowed for SDS PAGE isolation. The gel was silver stained to make the bands visible.

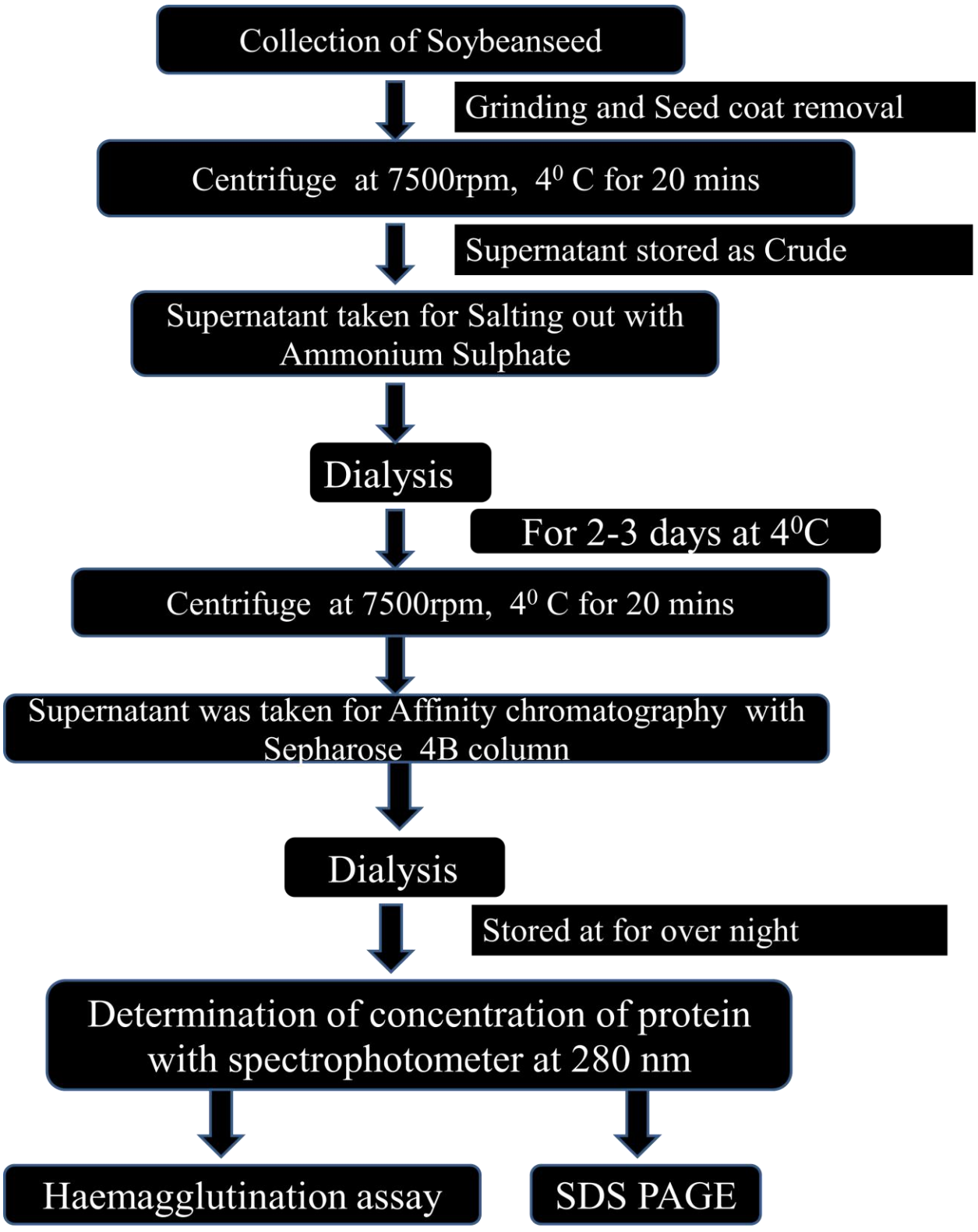

Fig.8: Protocol for Isolation of Lectin

6.RESULT:

6.1 PURIFICATION:

Soybean lectin was purified by affinity chromatography by Sepharose-4B column.

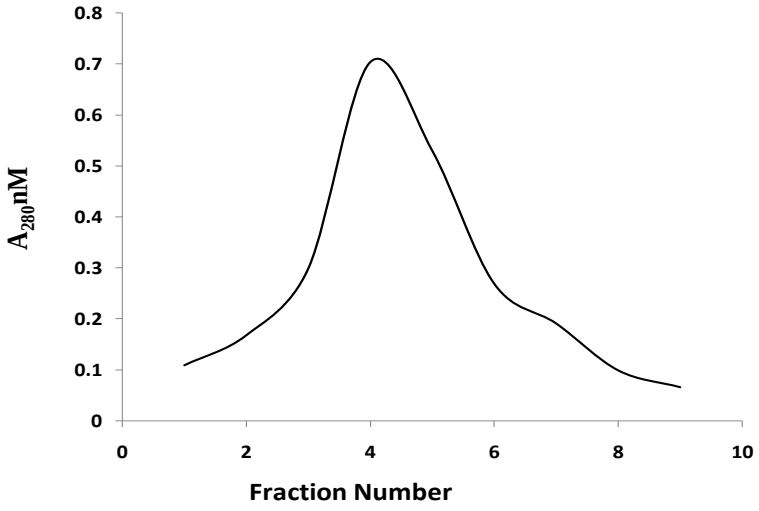

Fig.9: Elution of Profile of Soybean lectin from lactamyl-sepharose affinity matrix for 60% affinity

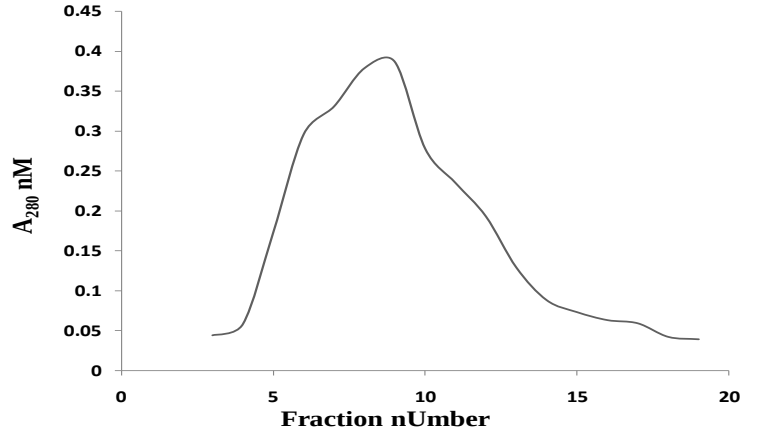

Fig.10: Elution of Profile of Soybean lectin from lactamyl-sepharose affinity matrix for 90% affinity

6.2 DETERMINATION OF PROTEIN CONCENTRATION :

Determination of the concentration of the proteins (crude, 20% cut, 60% cut,90% cut,60% affinity, 90%affnity) were done by Lowry method which is depicted in Table- 1.

Table-1: concentration of proteins:

Sample	Volume (ml)	Concentration (mg/ml)	Total concentration (mg/ml)
Crude	75	16.288	1221.6
20% cut	68	13.77	936.08
60% cut	57	12.76	727.07
90% cut	51	4.60	235.008
60%Affinity	44	0.327	14.3968
90%Affinity	36	0.167	6.0192

6.3. HAEMAGGLUTINATION ASSAY :

To characterize the desired protein, Haemagglutination Assay was performed by using human erythrocyte suspension of AB type blood groups. The assay was carried out in 96 well "U" bottom micro titer plate by serially diluting the sample and allowing it to incubate for 2 hours. Then it was found that there was agglutination reaction in the 90% affinity sample till 64 titer and in others RBCs were settled at the bottom.

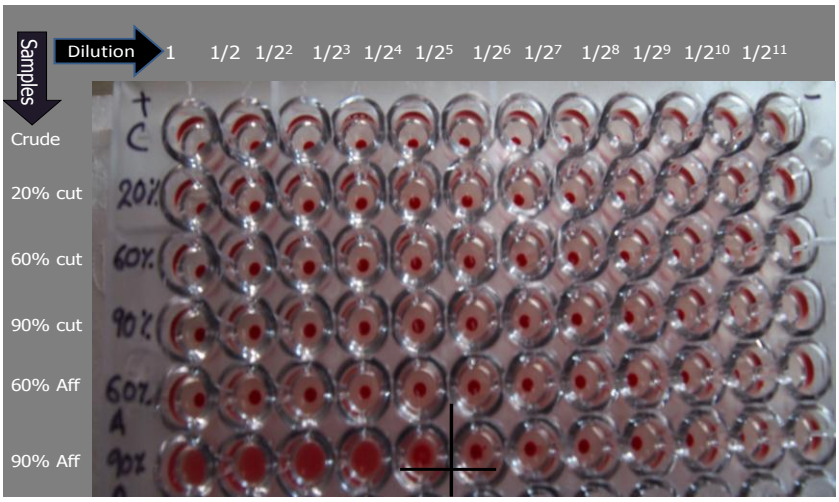

Fig.11: Haemagglutinatioon Assay for AB+ve Blood group

6.4 SDS- PAGE :

To determine the size of the protein, SDS-PAGE was performed using 12% polyacrylamide as the resolving gel and 5% polyacrylamide as the stacking gel and the bands were stained by silver staining method. Then the bands were visualized by gel documentation system and the molecular weight of the desired protein was found 30kD.

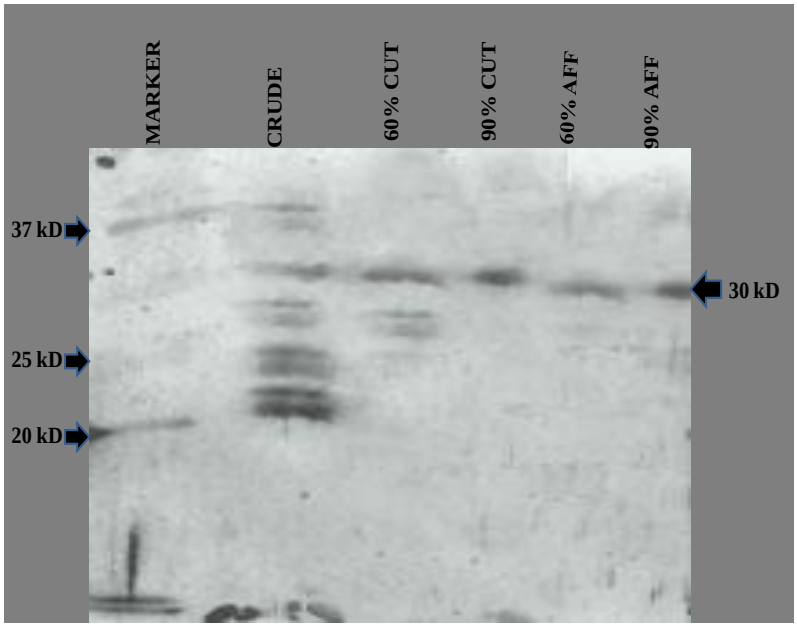

Fig.12: SDS-PAGE (12%) Electrophoretogram

7.DISCUSSION:

Many reports have been suggested about many structural and biochemical uses of plant lectins and their increasing use as biotechnological tools. According to early investigators the role of lectins in plant is defense against seed predators and during seedling development (Quinn et al., 1987; Naeemet al., 2001). Lectins are abundantly present in Legumes. In seeds, lectins accumulate in the protein bodies to form well-characterized storage proteins.

Lectins can be readily separated by size exclusion chromatography or by affinity chromatography (Sharon and Lis1972;). Spilatro and Anderson (1989) reported regarding isolation and purification of soybean lectin several studies. In 1952 Soybean lectin (SBL) was originally purified by Leiner and Pallansch and the same lectin was again purified on affinity column with immobilized amino caproyl-β-D- galactosylamine coupled to sepharose by Sharon and Lis (2004). In the present study the affinity elusion profile showed a single peak.

Lectins have the ability to agglutinate with the erythrocytes (Lis and Sharon, 1972). The eluted protein shows agglutinating activity when reacted with different types of fresh erythrocytes. In the present study, a lectin was isolated from soybean (SBL) seemed to bind to the erythrocytes. All steps of purification was Haemaggutination assayed to check lectin activity by measuring agglutination with the specifically prepared human red blood cells. The haemagglutination assay had primary importance since it determined the carbohydrate binding properties. Carbohydrate specificity of the lectin was examined by monitoring the displacement of the bound lectin from erythrocytes by various sugars. The lectin was found to agglutinate human erythrocytes of AB blood group which describes its non-specificity property.

The purified SBL obtained when treated with denaturing and reducing conditions in the presence of sodium dodecyl sulphate (SDS) and 2-β, mercaptoethanol according to Laemmli (1970), the lectin moved and a single band was found. In the present study the purified protein has band at a position of 30 kDa.

8.SUMMARY AND CONCLUSION:

In summary, SBL were successfully isolated and purified from the seed of Soybean (*Glycine max*) by affinity chromatography. The haemagglutination assay characterized the SBL which shows that these proteins could agglutinate with the human erythrocytes due to the presence of carbohydrate-binding site. The soybean lectin (SBL), agglutinates with the erythrocytes of AB type blood group. The SDS-PAGE of SBL showed the band at 30kDa.

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